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14. **ABSTRACT**
The Sprouty gene family negatively regulates growth factor-induced receptor tyrosine kinase signaling. I have demonstrated that Sprouty1 and 4 are down-regulated in human prostate cancers. The purpose of the present study is to elucidate the molecular mechanism(s) regulating Sprouty expression in prostate cancer. Results: I have carried out DNA methylation analysis on 20 matched normal prostate tissues and tumor prostate tissues (at least 70% of tissue is carcinoma) in the 5' untranslated region of Sprouty1 and 4 genes. Results show hypermethylation of the Sprouty4 in prostate cancer tissues; more than half of all prostate cancer DNAs were methylated in this region and methylation significantly correlated with decrease in Sprouty4 expression as determined by quantitative RT-PCR. Methylation analysis of Sprouty1 5' untranslated region is currently being investigated. To investigate the transcriptional regulation of Sprouty1 in prostate cancer, I have identified transcription start site(s) in 5'RACE reaction and characterized the Sprouty1 promoter region. Transient transfections using luciferase reporter gene constructs with progressive deletions of the human Sprouty1 5'-flanking region revealed that the core promoter activity is located within the proximal 0.3-Kb region. Comparative analysis of the 5'-flanking region of the human and mouse Sprouty1 shows a highly conserved binding site for Wt1, a transcription factor involved in renal development and tumorigenesis suggesting that Wt1 may be a key transcriptional regulator in Sprouty1 gene expression. Work is currently underway to identify the transcription factors binding to this core promoter region using a novel protein-DNA interaction based method. Conclusion: My studies suggest a potential tumor suppressor activity of Sprouty1 and 4 in prostate cancer. Complete elucidation of the molecular mechanisms controlling Sprouty expression may prove useful in understanding the regulation of growth factor signaling in prostate cancer.

15. SUBJECT TERMS
sprouty, growth factor & receptor tyrosine kinase signaling, prostate cancer, methylation, promoter, transcription factors.

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INTRODUCTION

Prostate cancer is the most common malignancy in US men and the second leading cause of cancer deaths. There is abundant evidence that increase fibroblast growth factor receptor signaling plays a critical role in the initiation and progression of prostate cancer (for review see [1]). Sprouty was originally identified in Drosophila as a negative regulator of fibroblast growth factor (FGF) signaling during tracheal development [2]. Subsequent studies have shown Sprouty to be a general inhibitor of growth factor-induced receptor tyrosine kinase (RTK) signaling pathways involved in Drosophila development and organogenesis [3-5]. While Drosophila has only one Sprouty gene, at least four Sprouty homologies (Sprouty1-4) have been cloned in human as well as mouse [2,6,7]. Mammalian Sprouty inhibit growth factor-induced cell responses, by inhibiting the RTK-dependent Ras/mitogen-activated protein (MAP) kinase signaling pathway [8-15] Several mechanisms for Sprouty inhibition of the RTK/Ras/MAP kinase pathway have been proposed, including blocking the interaction of the Grb2/SOS complex with FRS2 or Shp2 [3,14] or the inhibition of Raf [11,12]. Another characteristic of the Sprouty inhibitors is their regulation by growth factors in a negative feedback loop. Specifically, growth factors regulate both the level of Sprouty transcript [7] and in some systems, the recruitment of Sprouty proteins to the plasma membrane [16]. Furthermore, growth factors control Sprouty activity through the rapid and reversible tyrosine phosphorylation [17]. Importantly, each Sprouty family member is selectively tyrosine phosphorylated by a unique cohort of growth factors and with different kinetics, suggesting non-redundant functions for the Sprouty proteins [17]. Given that Sprouty proteins can inhibit FGF signal transduction, they can potentially decrease the biological activities of FGFs in prostate cancer cells and

inhibit their ability to promote cancer progression. I have previously shown by immunohistochemical and quantitative real-time PCR analysis that Sprouty1 is down-regulated in approximately 40% of prostate cancers when compared with normal prostate [18]. Down regulation of Sprouty2 mRNA has also been reported in breast cancer and a number of other common malignancies [19]. However, the molecular mechanism(s) regulating Sprouty expression in human cancers have not been characterized.

BODY

As outlined in my Statement of work, I seek to accomplish 3 main tasks during my 3 years of funding. I have made substantial progress on two of these tasks as described below. A manuscript describing the molecular mechanism regulating Sprouty4 expression in prostate cancer has been accepted for publication in the Prostate Journal. A copy of this manuscript is attached and will be referred to below.

Task2: Identification of the transcriptional elements that regulates human Sprouty1 expression (1-18 months).

Characterization of Sprouty1promoter region. The human Sprouty1 gene consists of two splice variants, 1a [2] and 1b [20] that maps to human chromosome 4q27-28 and 4q25-28 respectively. Each splice variant has 2 exons and one intron. Exon 1 encodes the 5'-untranslated region of the cDNA, whereas exon2 encodes the remainder of the 5'-untranslated region, the entire open-reading frame and the entire 3'-untranslated region. While the splice variants share the same second exon, they have different first exons, located very close to each other on the same chromosome (Figure 1).

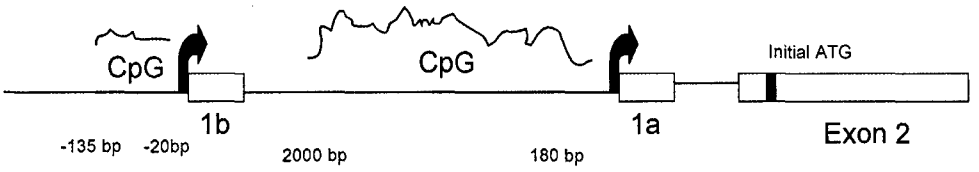


Figure1. Schematic representation of Sprouty1 gene. Exons are shown as open-boxes and translational start site, ATG is shown as thick black bar. CpG island in 5'UTR region of the splice variants 1a and 1b are shown. Promoter region is shown as black arrows. The use of alternative promoters does not result in protein isoforms because the variant 5' initial exons are Joined to a common second exon that contains the translation initiation site.

Identification of the transcription start sites of human Sprouty1 (1-3 months). I have used 5'RACE to localize the position of the human Sprouty1 transcription start site(s). 5'-RACE was performed using poly $(A)^+$ RNA from fetal human lung and a Sprouty1 specific primer. A strong single band of about 275 bp was obtained after amplification (data not shown). This 5'RACE product was subsequently subcloned and sequenced. Sequence analysis identified multiple transcription initiation sites within the region -315 to -305 nucleotides from the first ATG codon in a Kozak consensus sequence. The 5'-most start site found is located at nucleotide position 160026 of the published sequence (GenBank accession no. AC026402). Because this region corresponds to the 5'-UTR of Splice variant 1b, this must be the corresponding promoter region. Using similar approach I identified the transcription start site for Splice variant 1a to be at nucleotide position 162754 in the same published sequence (GenBank accession no. AC026402). These promoters are shown in the schematic diagram above (Figure 1).

Functional characterization of Sprouty1 promoter region (4-10 months). To localize the DNA elements that are important for promoter activity, I carried out a series of unidirectional deletion analyses of up to 2 kb 5'-flanking region of the Sprouty1a and Sprouty1b splice gene variants. Deletion fragments were generated by PCR and cloned into the promoterless pGL3-Basic, a luciferase reporter vector. Each resulting

recombinant construct was then transiently transfected into prostate cancer cell lines; LNCaP, PC3 and DU145 and normal immortalized prostate cell line pNT1A. After 48 h, cell extracts were prepared and luciferase activity was measured. Cells were co-transfected with a constant amount of pSV-β-galactosidase control plasmid as an internal control for transfection efficiency. The resulting luciferase reporter gene activities were then normalized to β-galactosidase activities. As shown in Figure 2, the promoter activity as determined by reporter gene expression showed significant difference between Splice 1a (A) and 1b (B) variants. Splice 1a promoter strength was between 2 to 5 fold above the basal level (A). Whereas Splice 1b promoter activity was between 40 and 1300 fold above basal level depending on the cell line (B). Furthermore, the reporter gene expression levels showed significant differences among the different prostate cell lines suggesting that cell-specific element(s) may be present in these sequence. Interestingly, the androgen-dependent cell line, LNCaP which expressed the least Sprouty1 protein expression level as determined by western blot analysis [18] showed the strongest promoter activity, expressing over 10 fold higher promoter activity than any of the other cell lines. The maximum promoter activity varied for each cell line, In LNCaP cells the maximum promoter activity was seen with the construct pSprouty1bFWD3 (-175 to +61). In the androgen independent prostate cancer cell lines PC3 and DU145, maximum promoter activity was seen with the construct pSprouty1bFWD4 (-233 to +61) and pSprouty1bFWD6 (-530 to +61) respectively. In the normal immortalized prostate cell line pNT1A, maximum promoter activity was seen with the construct pSprouty1bFWD5 (-305 to +61).

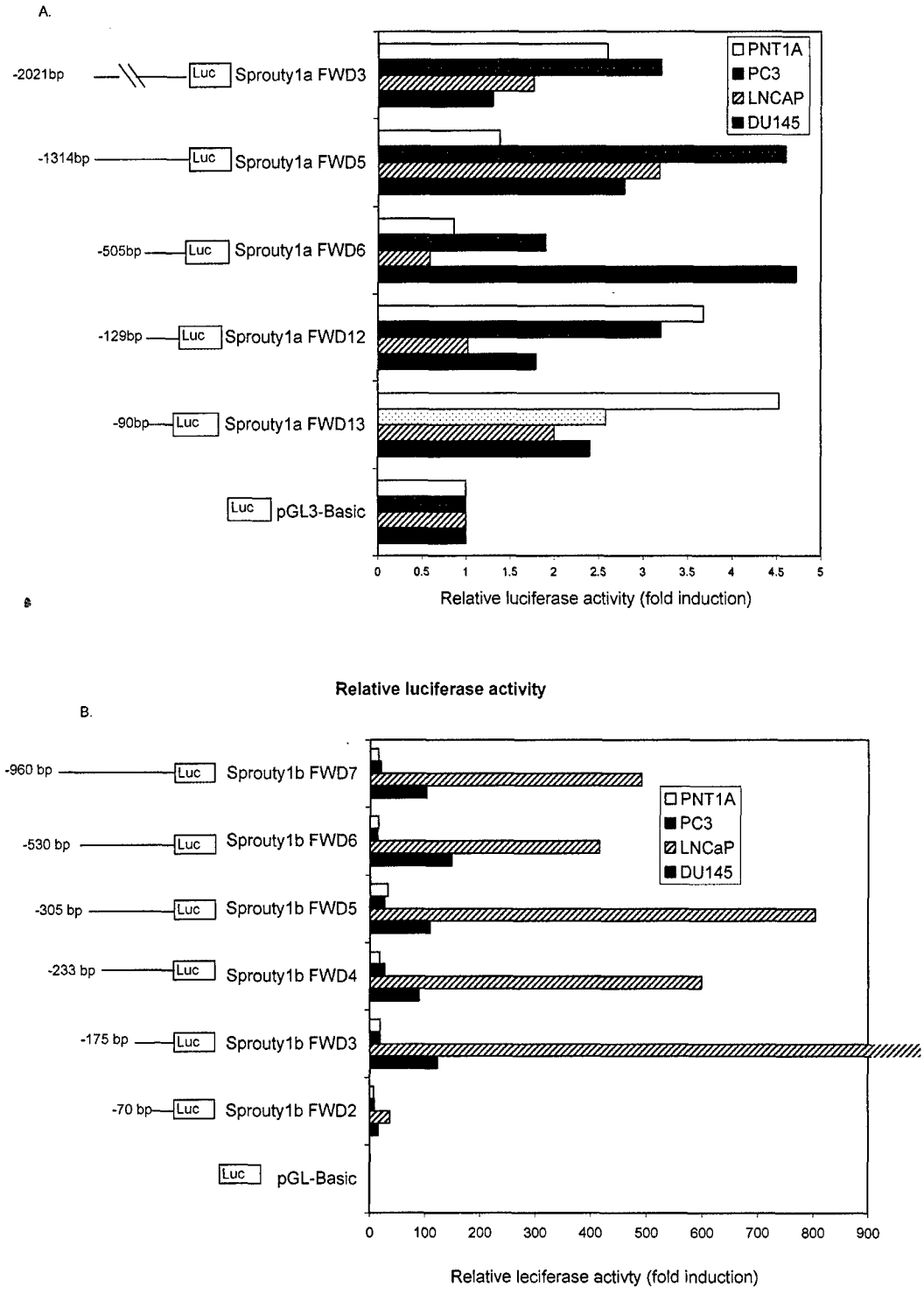


Figure 2. Progressive deletion analysis of the 5'-flanking region of splice variant 1a and 1b of the human Sprouty1 gene. The schematic diagrams represent a series of Sprouty1a (A) and 1b (B)

gene constructs with variable 5'-ends as indicated. The constructs were all cloned into a luciferase reporter vector (pGL3-Basic). 1.6 μg of each luciferase construct and 0.64 μg of the internal control pSV-β-galactosidase expression plasmid were transfected into LNCaP, DU145, PC-3 and pNT1A cells. Cells were lysed 48 hours post-transfection. The luciferase activity was measured and normalized for transfection efficiency by dividing the measurement of the firefly luciferase antivity by that of the β-galactosidase activity. The relative luciferase activities are represented as fold induction with respect that obtained in cells transfected with the empty control vector (pGL3-Basic).

Sequence analysis of the 5-flanking region of Sprouty1b promoter. In order to assess the promoter activity of the 5'-flanking region of the human Sprouty1b splice gene, I analysed up to 2 kb genomic AC026402 sequence upstream of the determined transcription start sites, using computer-based analysis (MatInspector software from Genomatix; www.Genomatix.de). Putative binding sites for transcription factors GATA1 (core similarity 1.000), EGR2 (core similarity 1.000), transcription repressor, ZBP (core similarity 0.761), ETS (core similarity 1.000), hypermethylated in cancer 1 transcriptional repressor, HIC (core similarity 1.000), E2F (core similarity 1.000), Elk-1 (core similarity 0.866) and FKHD (core similarity 1.000) were identified in the proximal sequence as shown in Figure 3. This analysis suggests that Sprouty1b promoter is likely regulated by multiple transcription factors.

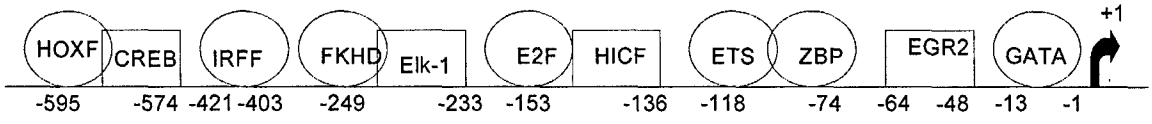


Figure 3. A schematic representation of of Sprouty1b proximal promoter region indicating the location of putative transcription factor binding sites. Transcription start site is shown as +1 and promoter indicated as a black arrow.

Comparative analysis of the 5'-flanking regions of the human and mouse Sprouty1 genes.

The human and murine Sprouty1 5'-flanking region upstream of their transcription start sites were aligned for sequence comparison. Over the entire 5'-flanking region upstream of the human Sprouty1 promoter, only a very short region in Splice variant 1b promoter, between -112 and +1 relative to the transcription showed approximately 94% degree of homology with the mouse Sprouty1 promoter. As illustrated in Figure 4, Wt1 transcription factor binding sites: EGR1 and 3 [21], and WTE [22] are conserved between the two species. This high homology indicates evolutionary conserved mechanism(s) involving Wt1 transcription factor in Sprouty1 gene regulation.

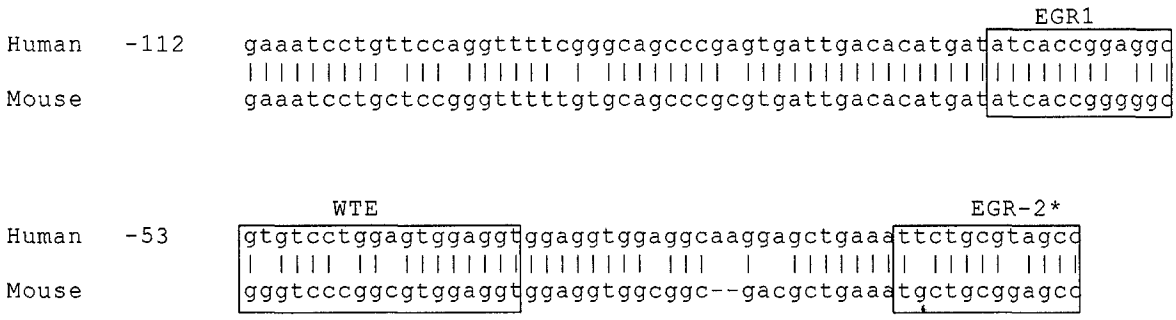


Figure 4. Alignment of sequence in the 5'-flanking region of human and murine Sprouty1 gene. The nucleotide sequences surrounding the transcription start site and the 5'-flanking region were compared. The putative binding sites for indicated transcription factors, which are conserved in both species, are boxed. An asterics (*) indicate core similarity of 1.000 with human sequence.

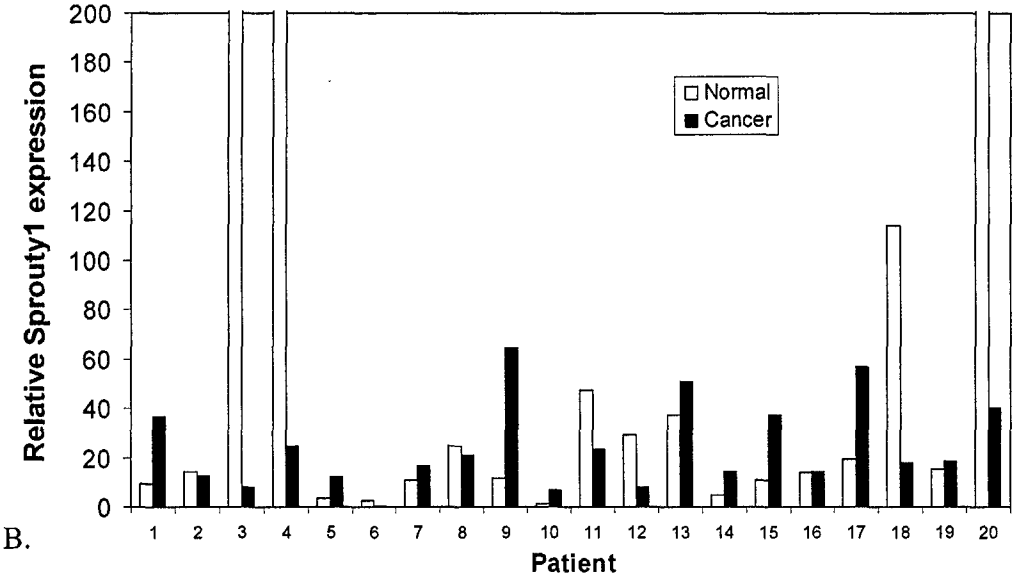
Task2.

Perform methylation analysis of the Sprouty1 promoter region in human normal prostate and prostate cancer in 30 DNA samples (6-10 months)

Using the MethPrimer software package for CpG islands identification (http://www.urogene.org/methprimer/), I have identified 2 separate CpG islands: 1 spanning about 2 kbp of 5'UTR region in splice variant 1a and the other spanning about

110 bp of 5'UTR region in splice variant 1b (see Figure1 above). This led me to investigate the possibility of epigenetic inactivation at this locus. Initially, I performed methylation analysis on the proximal Sprouty 1a 5'UTR region on 20 pairs of matched normal and tumor prostate tissue samples. The data shown in Figure 5B indicates DNA methylation in both the normal and tumor samples. Comparison of % methylation to Sprouty1 mRNA expression level in matched samples did not shown any significant correlation between Sprouty1 mRNA expression and DNA methylation (Figure 5A).

A.



B.

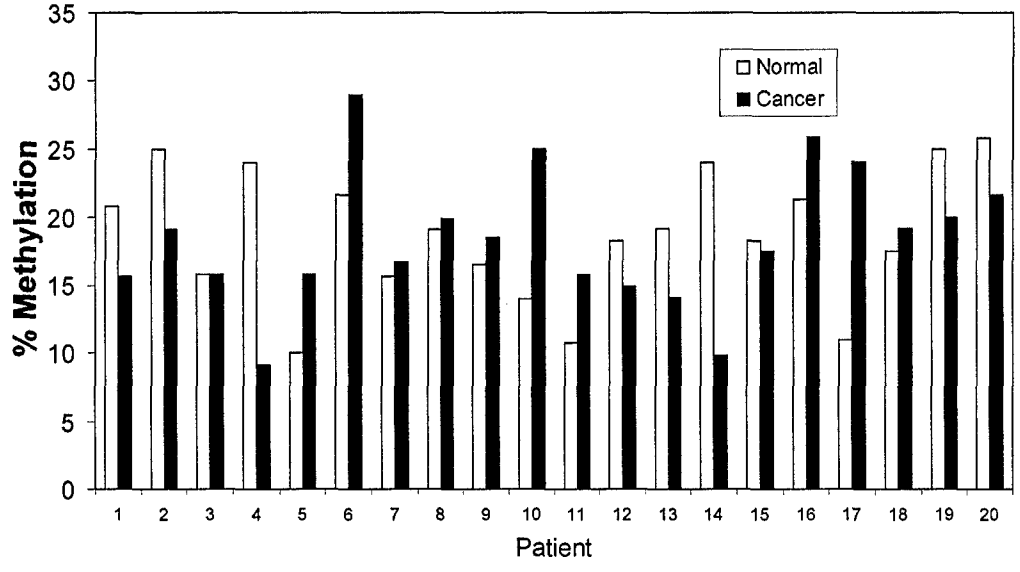


Figure5. Methylation analysis of Sprouty1a gene. A. Sprouty1 expression in normal prostatic tissues (□) and cancer tissues (■) was assessed by quantitative RT-PCR using a real-time thermal cycler (iCycler, Bio-Rad). Sprouty1 expression levels are displayed as a ratio of Sprouty1 transcripts X 10^3 to β-actin transcripts. The Sprouty1 and β-actin values were calculated from standard curves. B. Analysis of CpG methylation in bisulfite-converted genomic DNA derived from 20 normal prostate tissue samples (□) and 20 prostate cancer tissue samples (■). Frequency of methylation is shown as % methylation.

Treatment of prostate cell lines with 5-aza-2'deoxycytidine to restore Sprouty1 expression (10-12 months). I have previously measured Sprouty1 protein expression in normal prostatic epithelial cells and the prostate cancer cell lines LNCaP, PC3 and DU145 by western blot analysis [18]. I detected the highest Sprouty1 protein expression in the normal prostatic epithelial cells. PC3 and DU145 cell lines showed intermediate expression, whereas the Sprouty1 protein was essentially undetectable in LNCaP cells. To test the hypothesis that pharmacological modulation of methylation can reactivate gene expression [23], I treated immortalized primary prostatic epithelial cells, pNT1A and prostate cancer cell lines DU145, PC3 and LNCaP cells in various doses of the DNA methyltransferase inhibitor 5-aza-2'deoxycytidine (Aza dCR). Figure 6 indicates that treatment of the prostate cancer cell lines, DU145, PC3 and LNCaP with Aza dCR (2 μM) led to a significant increase in Sprouty1 mRNA expression in all the prostate cancer cell lines suggesting that promoter methylation is a key mechanism in the regulation of Sprouty1 expression in these cell lines. In support of this I (see attached manuscript) and others [24] have shown that DNA methylation is a key mechanism of Sprouty inactivation in prostate cancer.

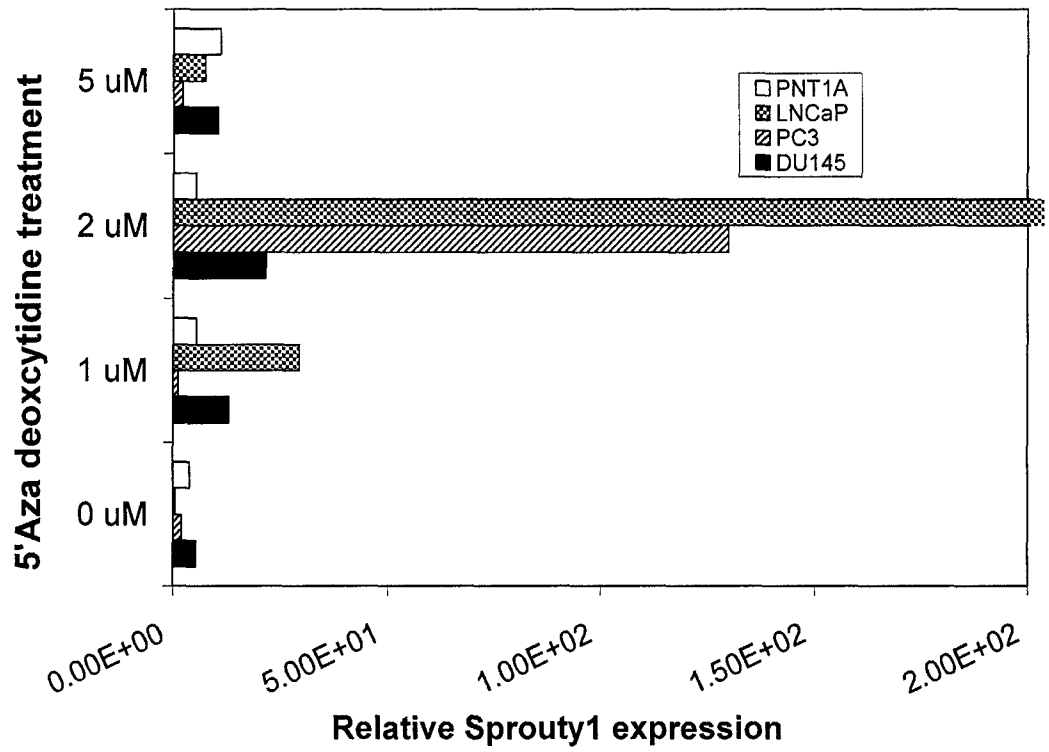


Figure 6. Demethylation and Sprouty1 expression. Prostate cancer cell lines; LNCaP, PC3 and DU145, and immortalized primary prostatic epithelial cells; pNT1A were treated with 5'-aza-2'-deoxycytidine (5'-aza-dC) at the indicated concentrations for 96 hours. Sprouty1 mRNA expression was determined by quantitative RT-PCR using iCycler and expressed relative to β-actin to correct for variation in the amounts of reverse-transcribed RNA. The data is a representative of duplicate experiments.

KEY RESEARCH ACCOMPLISHMENTS

- Identification and functional characterization of Sprouty1 promoter.
- A highly conserved binding site for WT1, a transcription factor involved in renal development and tumorigenesis may be a key transcriptional regulator of Sprouty1 gene expresseion.
- DNA methylation analysis suggests that epigenetic inactivation may be a key mechanism of Sprouty1 inactivation in prostate cancer.

REPORTABLE OUTCOMES

- **Manuscript accepted for publication in The Prostate Journal.**
- **Presentation- AACR Annual Meeting: Elucidating the functional regulation of Sprouty4, a growth inhibitor in prostate cancer. Kwabi-Addo et al., (2005) Anaheim, CA (Abstract).**

CONCLUSION

The Sprouty gene family functions as negative regulators of receptor tyrosine kinase signaling. I have shown that Sprouty1 and 4 are down-regulated in the majority of human prostate cancer. My studies have indicated that the mechanisms by which Sprouty1 and 4 is down-regulated in prostate cancer is due, at least in part by epigenetic inactivation. Complete elucidation of the molecular mechanisms controlling Sprouty expression may prove useful in understanding the regulation of growth factor signaling in prostate cancer which may in turn provide an attractive new target approach for therapeutic intervention that may modulate a large number of potential growth promoting stimuli, including multiple growth factors and their receptors.

Future work will focus on :

1) Completing the evaluation of Sprouty1 gene inactivation in human prostate cancer by performing methylation analysis of Sprouty1b promoter region, using quantitative RT-PCR and Southern blot hybridization analysis to evaluate for hemizygous and/or homozygous deletion of the Sprouty1 locus using 30 DNA samples highly enriched for prostate carcinoma.

2) Completely characterize the transcription factors responsible for Sprouty1 gene regulation by identifying transcription factors that binds to Sprouty1 promoter using TranSignal Protein/DNA arrays and electrophoretic mobility shift assays. Use site-directed mutagenesis to assess the effects of mutating potential transcription factor binding sites on Sprouty1 transcription and evaluate specificity of transcription factor binding interaction in and investigate in vivo molecular interaction of transcription factors and Sprouty1 promoter.

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4623 Elucidating the molecular mechanisms regulating the expression of Sprouty4, a growth inhibitor in prostate cancer

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A considerable body of evidence indicates that fibroblast growth factors (FGFs) and their receptors are key modulators of normal prostate proliferation with their over-expression associated with aggressive prostate cancer (PCa) and poor survival outcome. Recent studies have identified a new family of regulators of FGF activity. The Sprouty gene family negatively regulates FGF signaling in a variety of systems and could potentially limit the biological activity of FGFs in prostate cancer. Using quantitative real-time polymerase chain reaction (QRT-PCR), we have found that the expression of human Sprouty4 mRNA is down-regulated in PCa when compared to normal tissues where as FGF2 mRNA expression in the same set of tissue samples was 2 fold higher in PCa than normal prostate tissues. The decrease in Sprouty4 expression implies a loss of an important growth regulatory mechanism that may potentiate the effects of increased FGF expression in PCa. We sort to understand the molecular mechanisms regulating Sprouty4 expression in PCa. The human Sprouty4 gene maps to the long arm of chromosome 5 and inactivation of this chromosomal region is a frequent event in PCa. We therefore investigated whether human Sprouty4 is a likely target for gene inactivation in PCa. The presence of a large CpG islands from -1200 nt upstream of the putative transcription start through the first exon into the intronic sequence led us to investigate the possibility of epigenetic inactivation at this locus. Using methylation specific PCR in conjunction with sequencing, we have observed extensive methylation of the Sprouty4 CpG island in a subset of cancer cases when compared to normal peripheral tissue samples. Treatment of prostate cancer cell lines with 5'-aza 2'-deoxycytidine restored Sprouty4 gene expression confirming that methylation caused the gene down-regulation. In summary, our data suggests that DNA methylation is a likely mechanism for the down-regulation of Sprouty4 expression in prostate cancer. Complete mutational and LOH analysis, as well as gene and protein expression studies, needs to be performed to fully elucidate the molecular mechanisms regulating Sprouty4 expression in prostate cancer.

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The Prostate 9999:1–12 (2005)

Sprouty4, a Suppressor of Tumor Cell Motility, is Downregulated by DNA Methylation in Human Prostate Cancer

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PURPOSE. Alterations of fibroblast growth factors (FGFs) and their receptors contribute to prostate cancer progression by enhancing cellular proliferation, survival, and motility. The Sprouty gene family negatively regulates FGF signaling and may limit the ability of FGFs to enhance tumor progression. Sprouty1 is downregulated in human prostate cancers and Sprouty1 expression can markedly inhibit prostate cancer proliferation in vitro. Sprouty4 has been shown to negatively regulate both proliferation and cell migration in other systems. We therefore examined whether Sprouty4 expression was altered in prostate cancer.

EXPERIMENTAL DESIGN. Expression of Sprouty4 was examined by in situ hybridization and quantitative RT-PCR. Methylation of the Sprouty4 gene promoter was assessed using bisulfite modification and sequencing. The effect of Sprouty4 expression on cell migration was determined using an in vitro wounding assay.

RESULTS. By in situ hybridization Sprouty4 is expressed in normal prostatic epithelial cells and is decreased in a subset of prostate cancers. Quantitative RT-PCR confirms that Sprouty4 expression is decreased in approximately one half of prostate cancers. Analysis of the 5′-regulatory region revealed a CpG island approximately 1 kb upstream of the transcription initiation site, the proximal portion of which was preferentially methylated in prostate cancer tissues. More than one half of all prostate cancer DNAs were methylated in this region and methylation was significantly correlated with decreased Sprouty4 expression as determined by quantitative RT-PCR. When overexpressed in prostate cancer cell lines, Sprouty4 did not inhibit cell proliferation but did inhibit cell migration.

CONCLUSIONS. Sprouty4 expression is downregulated in human prostate cancer by DNA methylation and this decreased expression may contribute to increased cell migration. *Prostate 9999: 1–12, 2005.* © 2005 Wiley-Liss, Inc.

KEY WORDS: **Sprouty; prostate cancer; methylation; cell migration; growth factor**

INTRODUCTION

Prostate cancer is the most common malignancy in US men and the second leading cause of cancer deaths. There is abundant evidence that increased fibroblast growth factor receptor signaling plays a critical role in the initiation and progression of prostate cancer (for review see Kwabi-Addo et al. [1]). Fibroblast growth factors (FGFs) have multiple biological activities in prostate cancer including increasing proliferation [2], angiogenesis [3], and cell motility [4] and as well as inhibiting cell death [5], all of which can promote tumor

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progression. In normal tissues, FGF signaling can be controlled by a number of mechanisms. Sprouty was originally identified in *Drosophila* as a negative regulator of fibroblast growth factor signaling during tracheal development [6]. Subsequent studies have shown Sprouty to be a general inhibitor of growth factor-induced receptor tyrosine kinase (RTK) signaling pathways involved in *Drosophila* development and organogenesis [7–9]. While *Drosophila* has only one Sprouty gene, at least four Sprouty homologues (Sprouty 1–4) have been cloned in human as well as mouse [10,11]. Mammalian Sprouty proteins inhibit growth factor induced cell responses, by inhibiting the RTK-dependent Ras/mitogen-activated protein (MAP) kinase signaling pathway [12–19]. Several mechanisms for Sprouty inhibition of the RTK/Ras/MAP kinase pathway have been proposed, including blocking the interaction of the Grb2/SOS complex with FRS2 or Shp2 [7,12] or the inhibition of Raf [16,17]. Another characteristic of the Sprouty inhibitors is their regulation by growth factors in a negative feedback loop. Specifically, growth factors increase the level of Sprouty transcript [11] and in some systems, the recruitment of Sprouty proteins to the plasma membrane [20]. Furthermore, growth factors control Sprouty activity through the rapid and reversible tyrosine phosphorylation [21]. Importantly, each Sprouty family member is selectively tyrosine phosphorylated by a unique cohort of growth factors and with different kinetics, suggesting non-redundant functions for the Sprouty proteins [21]. Recently, Sprouty4 was shown to inhibit the kinase activity of the testicular protein kinase 1, TESK1 by binding to it through the C-terminal cysteine-rich region [22,23]. TESK1 is a serine/threonine kinase that phosphorylates cofilin and plays a role in integrin-mediated actin cytoskeletal reorganization and cell spreading [24–27]. Although tyrosine phosphorylation is required for the inhibitory activity of Sprouty4 on a Ras/MAP kinase pathway, mutation of the corresponding tyrosine (Tyr-75 in human Sprouty4) to alanine had no apparent effect on its inhibitory actions on TESK1 activity and cell spreading, suggesting a novel cellular function of Sprouty4 to regulate the actin cytoskeleton, independent of it's inhibitory activity on the Ras/MAP kinase signaling.

Given that Sprouty proteins can inhibit FGF signal transduction, they can potentially decrease the biological activities of FGFs in prostate cancer cells and inhibit their ability to promote cancer progression. We have previously shown by immunohistochemical and quantitative real-time PCR analysis that Sprouty1 is downregulated in approximately 40% of prostate cancers when compared with normal prostate [28]. Downregulation of Sprouty2 mRNA has also been reported in breast cancer and a number of other common malignancies [29]. In the present study, we demonstrate that Sprouty4 is also downregulated in prostate cancer. The downregulation of these Sprouty isoforms in human prostate cancer implies a loss of an important regulatory mechanism in prostate cancers that may potentiate the effects of increased FGFs and FGF receptor expression in prostate cancer. In this report, we demonstrate the extensive methylation of a Sprouty4 CpG island in the majority of cancer cases when compared to normal peripheral tissue samples, which correlated with decreased Sprouty4 expression. In addition, treatment of the LNCaP prostate cancer cell line, in which this CpG island is methylated, with 5′-aza 2′-deoxycytidine restored Sprouty4 gene expression, confirming that methylation caused the gene downregulation. Furthermore we demonstrate that Sprouty4, unlike Sprouty1, does not cause cell growth inhibition but rather inhibits cell migration, suggesting that Sprouty1 and 4 perform different functions in prostate cancer. These observations support the idea that different Sprouty isoforms have distinct functions as tumor suppressors in prostate cancer and that expression of one or more Sprouty genes is decreased in most prostate cancers.

MATERIALS AND METHODS

Plasmid Construction

Plasmid pcDNA3.1 (Invitrogen, Carlsbad, CA) was used for the expression of full length Sprouty4 cDNA. For the construction of pcDNA-Sprouty4, the full coding sequence of Sprouty4 was amplified from EGFP-tagged hspry4 cDNA [23] (a gift from Onno C. Leeksma) in a PCR reaction using primers designed against the published Sprouty4 sequence as follows: forward primer 5′-AAGCTTAGACATGCTC AGCCCCC TCC-3′ and a reverse primer 5′-*GAATTC*CTAGAAAGGCTTGTCGGG-3′ (the underlined sequence indicates *Hind* III and *EcoR*I sites in the forward and reverse primers, respectively; bold and underlined shows the start and stop sites in the forward and reverse primers respectively; italicized and underlined G indicates an engineered G at position -3 to ensure proper initiation of translation). The PCR product was digested with *EcoR*I and *Hind* III and sub-cloned into pcDNA3.1. The pcDNA-Sprouty4 construct was verified by restriction digestion analysis and sequencing.

In Situ Hybridization of Sprouty4 in Prostate Tissues

The full-length Sprouty4 cDNA was cut from pcDNA-Sprouty4 (described above) and cloned as an *EcoR*I and *Hind* III fragment into pCMV-tag2B vector (Invitrogen). The universal primer T3 and T7 sequences in pCMV-tag2B were used to generate sense and

anti-sense RNA probes. Digoxigenin-labeled (Dig-labeled) anti-sense and sense RNA probes were synthesized using MAXIscript™ in vitro RNA transcription kit (Ambion, Inc., Austin, TX) with the linearized Sprouty4 plasmid as a template. A tissue microarray slide containing 14 prostate tissues was used for the in situ hybridization. Briefly, prostate tissues were dewaxed in xylene for 10 min (three times) and hydrated. Tissue sections were digested with 40 μg/ml proteinase K for 7 min at room temperature and then fixed in 4% paraformaldehyde for 20 min. The Dig-labeled probe (1 μg) was then added to 1 ml hybridization buffer (50% formamide, 10% SSC). Hybridization was performed at 70°C overnight, after which slides were sequentially washed by Dig Wash and Block Buffer Set (Roche Diagnostics, Indianapolis, IN) according to the manufacture's instruction. Antibody (1:2,000) against digoxigenin was used to detect the signal and NBT/BCIP was used as substrate for color development (Boehringer Mannheim, Germany). The staining in tissues was scored as no staining, weak staining, moderate, or strong staining.

Human ProstateTissue Samples

All samples of human prostate tissues were obtained with informed consent and maintained by the Baylor Specialized Program of Research Excellence (SPORE) in the prostate cancer tissue bank [30]. Fresh frozen tissue punches of normal and tumor tissue were obtained at the time of radical prostatectomy. The pathological status was confirmed before processing, and the tumor samples had a tumor cell percentage of 70%–100% with Gleason scores of 6–8. Formalin-fixed, paraffin-embedded specimens were also obtained from the Baylor SPORE prostate cancer tissue bank.

Bisulfite Modification and Sequencing

The methylation status of the Sprouty4 gene 5′-flanking CpG islands was analyzed by bisulfite treatment, which converts unmethylated cytosines to uridines (then thymidines) while retaining methylated cytosines as unchanged nucleotides, followed by PCR amplification, cloning, and sequencing. Briefly, DNA samples prepared from prostate tissues were modified by sodium bisulfite treatment using MethylEasy kit (Human Genetic Signatures, Sydney, AUS) according to the manufacturer's protocol. The modified DNA samples were used in PCR analysis with primer pairs designed using MethPrimer software package for methylated and unmethylated CpG islands identification (http://www.urogene.org/methprimer/). Primers used for the analysis of the proximal CpG island of the promoter were forward 5′-GTTTTTGGTGGAGTTT-GAGTTAGTT-3′ and reverse 5′-CCACTACCTAAA-AAAA TAAC TTTTT-3′; for the analysis of the distal CpG island of the promoter were forward 5′-GGTTT-TATTTATTTATTTGGTTAGTTTT-3′ and reverse 5′-TAAATATCCTTT CTCTATCCCAATC-3′. The PCR amplification step was as follows: 95°C for 3 min, then denature at 95°C for 30 sec, anneal at 58°C (proximal CpG island) or 60.8°C (distal CpG island) for 30 sec, extension at 72°C for 30 sec for 35 cycles, and a final 10 min extension at 72°C. The PCR product was subsequently cloned into pCR 2.1-TOPO vector using a TOPO TA cloning kit (Invitrogen) according to the manufacturer's instruction and the recombinants were sequenced using M13 reverse and T7 universal primers.

Cell Culture

The human prostate cancer cell lines, PC3, DU145, and LNCaP were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (Invitrogen). The human vascular endothelial cells (HUVEC) were cultured in complete EGM-2 medium (Cambrex Bioscience; Walkersville, MD).

Cell Transfection

For stable transfection, DU145 cells were seeded at 5×10^6 cells per 100 mm dish and transfected with 2.4 μg of Sprouty4 construct (pcDNA-Sprouty4) or vector only (pcDNA3.1) using Lipofectamine 2000 transfection reagent (Invitrogen) and according to the manufacturer's protocol. Two days after transfection, cells were selected in Geneticin (Sigma, St. Louis, MO) containing medium at a final concentration of 250 μg/ml. After 14 days into the selection Geneticin resistant clones were pooled together and propagated. For transient transfection, LNCaP, DU145, PC-3, and HUVEC were plated at 5×10^4 cells per 60 mm dish and transfected with 2 μg of Sprouty4 plasmid or vector only using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturers protocol. After 24, 48, or 72 hr cells were trypsinized and counted using a Coulter counter. A second transfected plate was used to collect protein extract for Western blotting at the same time.

Preparation, Quantification, and Dilution of DNA Standards

The Sprouty4 plasmid, keratin 18 plasmid (ATCC #MGC-9348) and β-actin plasmid (ATCC #MGC-10559) were prepared using the Qiagen Maxi-prep Kit (Qiagen, Valencia, CA). The Spred2 plasmid was constructed by amplifying the Spred2 coding sequence from LNCaP cDNA in a standard PCR reaction using primers designed as forward 5′-AGACGATGACAGC

TATATTGTGCGT-3′ and reverse 5′-TCTCGTCGCT-AGTATCGCACG-3′. The PCR product was cloned into pCR 2.1 TOPO vector using a TOPO TA cloning kit (Invitrogen) according to the manufacturer's instruction. The FGF2 plasmid have been previously described [31]. Quantification of plasmid was performed spectrophotometrically. The measurements of the plasmid concentration were done in duplicate and then converted to copy number. A dilution series of each plasmid (10^9 to 10^1 copies) was used as a DNA standard for real-time PCR.

Primer Design and Synthesis for Real-Time PCR

Oligonucleotide primers for Sprouty4 were forward 5′-TGACCAACGGCTCTTAGAC-3′; and reverse 5′-GCATTTACACTTCCCACAGG-3′; for keratin 18 were forward 5′-AGGGCTCAGATCTTCGCAAAT-3′ and reverse 5′-GTCATCAATGACCTTGCGGAG-3′; for β-actin were forward 5′-AGCACGGCATCGTCACCA-ACT-3′ and reverse 5′-TGGCTGGGGTGTTGAAGGT CT-3′; for Spred2 were forward 5′-TGAGCTTG-GCGATGATGAC-3′ and reverse 5′-CGAGGTGA-TAGTGGTCTGTG-3′ and for FGF2 were forward 5′-CCACTTCAAGG ACCCCAAG-3′; and reverse 5′ATAGCCAGGTAACGGTTAGC-3′. Primers were carefully designed to cross exon/intron regions, avoid the formation of primer-dimer, hair pin, and self complementarity. The nucleotide positions for the amplification products as given per the Genbank accession numbers are 577–757, 256–435, 458–622, 607–779, and 539–721 for Sprouty4 (AF227516), β-actin (BC004251), keratin 18 (BC020982), Spred2 (NM_181784), and FGF2, respectively.

cDNA Synthesis and Quantitative Real-Time PCR

Total RNA extracted from cells and tissues using TRIzoL Reagent (Invitrogen) was used in first strand DNA (cDNA) synthesis using Invitrogen Super-Script™ first strand synthesis system for RT-PCR and according to the manufacturer's protocol. Real-time PCR was carried out in a Bio-Rad iCycler real-time thermal cycler (Bio-Rad, Hercules, CA) as described previously [32] and incorporating the following optimized PCR reaction conditions: The amplification of Sprouty4 or Spred2 was carried out as follows: a 3 min hot start at 95°C followed by 40 cycles of denaturation at 95°C for 15 sec, and annealing at 61°C for 30 sec. The amplification protocol for FGF2 was the same as Sprouty4 except annealing was done at 63.5°C. The amplification protocol for β-actin or keratin 18 was carried out as follows: a 3 min hot start at 95°C, followed by 40 cycles of denaturation at 95°C for 30 sec, annealing at 56°C for 20 sec, and a 72°C extension for 30 sec. Each experiment was done in duplicate. The C_t values in log linear range representing the detection threshold values were used for quantitation and expressed as copy numbers based on a standard curve generated using plasmid DNA.

Western Blotting

Total protein was extracted from cells using protein lysis buffer as described previously [33]. For Western blots, 30 μg of protein extract/lane were electrophoresed, transferred to nitrocellulose membrane (Hybond™ ECL™, Amersham Pharmacia Biotech, Picataway, NJ) and incubated overnight with a 1:2,000 dilution of anti-Sprouty4 rabbit polyclonal antibody (Upstate Biotech, Waltham, MA) or a 1:5,000 dilution of anti-β-actin mouse monoclonal antibody (Sigma). Membranes were washed and treated with goat anti-rabbit IgG (1:5,000; Bio-Rad) or rat anti-mouse IgG secondary antibody conjugated to horseradish peroxidase (1:2,000 dilution; Southern Biotechnology Associates, Birmingham, AL) for Sprouty4 and β-actin, respectively. The antigen-antibody reaction was visualized using an enhanced chemiluminesence (ECL) assay (Amersham) and exposed to ECL film (Amersham).

Induction of Sprouty4 Expression by 5′-Aza-2′-Deoxycytidine (5′-aza-dC)

LNCaP cells were seeded at 5×10^5 cells/100-mm tissue culture dish. After 24 hr of incubation, the culture media was changed to media containing 5′-aza-dC for 96 hr. Cells were then harvested for RNA extraction and the extracted RNA used in real-time quantitative PCR as described above.

Wounding Assay of Scatter/Migration

Prostate cancer cells were seeded at 2×10^6 in 60-mm diameter culture dished and grown to confluence in complete medium and analyzed using a classical scratch wound method. Cells were gently scraped with a plastic tip. The medium was removed, and cells were washed twice with PBS. Complete medium was added and cells were allowed to scatter/migrate into the area of clearing for a total of 48 hr and photomicrographs taken at 0, 24, and 48 hr time points.

RESULTS

Expression of Sprouty4 in Normal and Neoplastic Human ProstateTissue

We initially investigated the expression of the Sprouty4 gene in human prostate tissues. To achieve this, we used in situ hybridization for the detection of Sprouty4 in 14 prostate tissues samples, since currently

available antibodies are not suitable for immunohistochemistry (unpublished observation). In normal prostate tissues we observed Sprouty4 expression in the epithelium (Fig. 1a) with minimal expression in prostatic stromal cells. Sprouty4 expression was variable; some normal tissues showed moderate expression (Fig. 1a) while others showed strong expression (Fig. 1b,c). Prostate cancer cells also had quite variable expression. Many prostate cancer tissues showed weak expression of Sprouty4 (Fig. 1b–d). Other cancers had moderate or even strong expression of Sprouty4 (Fig. 1e). Interestingly in some samples where we observed moderate or strong expression of Sprouty4 expression in the normal cells, the adjacent cancer cells showed less Sprouty4 expression (Fig. 1b,c). In situ hybridization with sense probe gave no detectable signal (Fig. 1f). To quantitatively compare the expression of Sprouty4 in normal and neoplastic prostatic epithelium, we used quantitative real-time PCR analysis to determine the expression level of Sprouty4 mRNA in a total of 25 pairs of matched normal and tumor prostate tissue samples. We used β-actin as an endogenous mRNA control. The real-time data is presented as the ratio of Sprouty4 mRNA transcripts X 10^3/β-actin transcript for the samples analyzed (Fig. 2a). The expression of Sprouty4 in both normal prostate and cancer tissues was variable, presumably reflecting both random variability in tissue composition (i.e., epithelial content) and variable expression per cell. However, Sprouty4 expression was about fivefold higher on average in the normal prostate tissues (14.5 ± 12.5, SEM) compared to prostate cancers (3.10 ± 0.962, SEM). Examination of paired normal versus cancer tissues revealed decreased Sprouty4 expression in 11 out of 25 cancer cases (44%) relative to matched normal tissues. This is similar to the decrease in Sprouty1 expression in normal versus cancer cells as determined by immunohistochemistry of tissue microarrays [28]. Since Sprouty-4 is expressed almost exclusively in the epithelium, we also normalized Sprouty4 expression

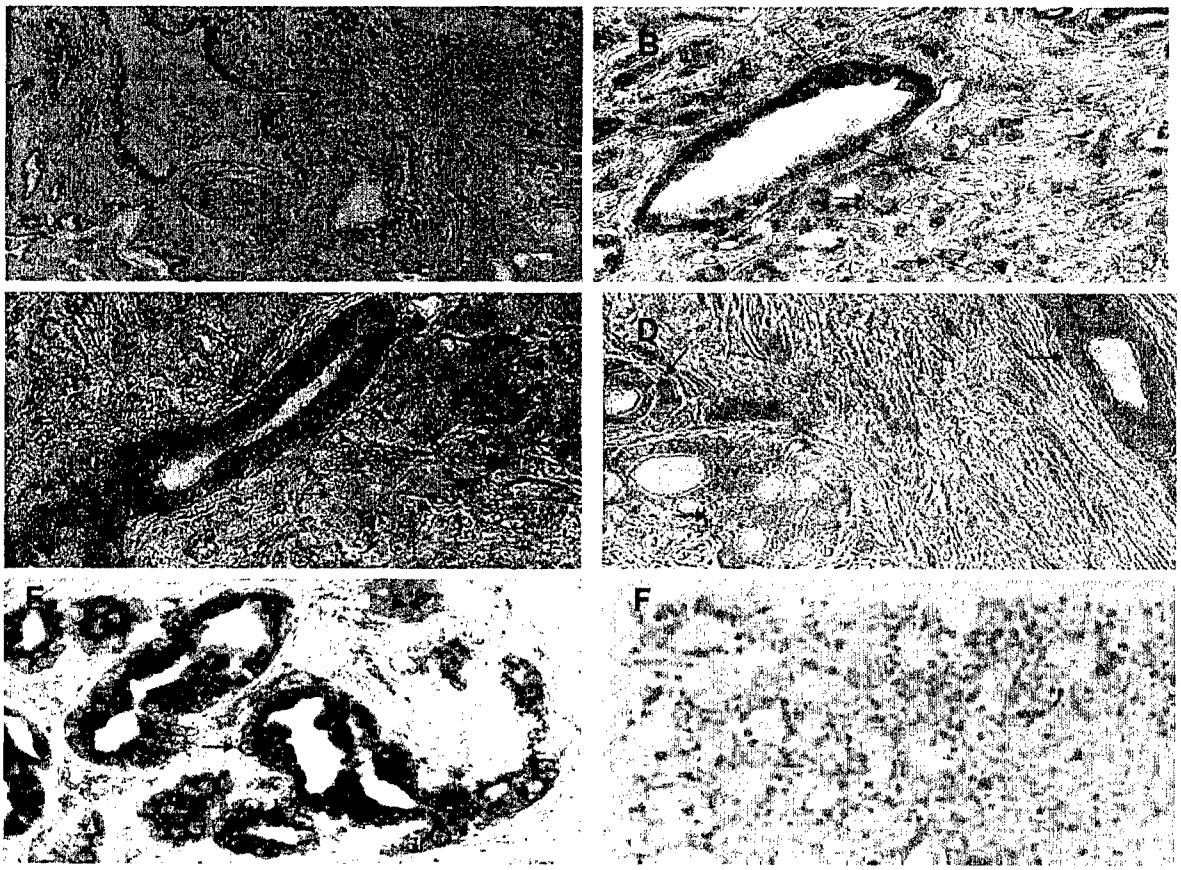

Fig. 1. In situ hybridization analysis of Sprouty4 expression in prostate tissues. Expression of Sprouty4 in normal prostate (**A**) and prostate cancer (**B–F**) was determined using in situ hybridization as described in Materials and Methods. A: Normal prostate peripheral zone tissue with expression of Sprouty4 in prostatic epithelial cells (long arrows). B–C: Normal prostate with strong expression of Sprouty4 (long arrows) with weak expression of Sprouty4 in diffusely infiltrating cancer cells surrounding normal tissue (some of which are indicated by short arrows). D: Prostate cancer glands with low Sprouty4 expression (short arrows). E: Prostate cancer with strong Sprouty4 expression in cancer glands (short arrow). F: Prostate tissue hybridized with sense probe.

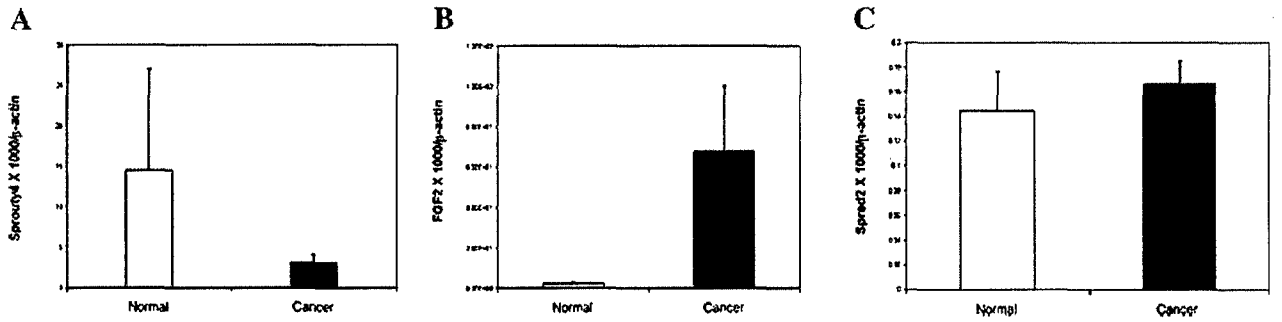

Fig. 2[Q2]. Quantitative RT-PCR to determine the mRNA expression of Sprouty4, FGF2 and Spred2 in matched normal and cancer prostate tissue samples. Gene expression in normal prostatic tissues and cancer tissues was assessed by quantitative RT-PCR. Gene expression levels are displayed as a ratio of transcripts $\times 10^3$ to β-actin transcripts. The specific gene and β-actin values were calculated from standard curves. The data is a representative of duplicate experiments. The mean expression level (±SEM) is indicated. **A**: Sprouty4 expression (**B**) FGF2 expression (**C**) Spred2 expression.

using keratin 18 mRNA, which is expressed only by epithelium, and as such may be a more relevant gene for normalization. Out of a total of 25 pairs of matched normal and tumor prostate tissue samples, 16 cancer samples revealed decreased Sprouty4 expression (64%) relative to the matched normal tissue (data not shown). One possible explanation for decreased expression of Sprouty4 mRNA in the cancer tissues is decreased expression of FGFs in a subset of the prostate cancers. We have previously shown that FGF2 and FGF7 are expressed in the stromal cells of cancer tissues and that FGF2 protein is approximately 2.5-fold higher in prostate cancer tissues, while FGF7 protein levels are similar in normal and prostate cancer tissues [33]. We therefore investigated FGF2 mRNA expression in the 25 pairs of matched normal and tumor prostate tissue samples using quantitative RT-PCR (Fig. 2B). We found that the FGF2 mRNA expression level was more than 20-fold higher on average in this set of prostate cancer samples (67.8 ± 6.4, SEM) compared to normal prostate tissues (2.57 ± 0.49, SEM; Fig. 2B). This data clearly demonstrates that the decrease in Sprouty4 expression in the prostate cancer tissues does not reflect decrease of FGF ligands. Finally, we examined the expression of Spred2 mRNA in prostate cancer and normal prostate tissue. The Spred genes are related to Sprouty and also inhibit Ras/Raf signaling and activation of MAP kinases [34]. As shown in Figure 2c, there was no significant downregulation of Spred2 mRNA in prostate cancer. Thus, the downregulation of Sprouty4 mRNA appears to be specific, since not all negative regulators of FGF signaling are decreased in prostate cancer.

Epigenetic Analysis of Sprouty4 Gene 5′-Flanking Region

The human Sprouty4 gene maps to the long arm of chromosome 5 and is approximately 14.5 kb in length interrupted by two introns. Exon1 only encodes the 5′-UTR of the cDNA, whereas exon 2 contains the translation initiation codon. The remainder of the open-reading frame for the protein as well as the entire 3′-UTR is encoded by the third exon. Multiple transcription start sites have been identified by 5′-RACE analysis [35]. The 5′-flanking region of the human Sprouty4 gene lacks a canonical TATA box or CAAT sequence within the expected proximity of the transcription start site. Transient transfection studies by this group revealed the presence of the maximal basal promoter activity within the 1,182-bp 5′-flanking region upstream from the transcription initiation sites [35]. As further evidence demonstrating the importance of this core promoter region, a comparison of human and murine Sprouty4 proximal promoter sequences showed significant homology with each other. We therefore investigated whether aberrant gene methylation of Sprouty4 in this region is a mechanism of downregulation in of Sprouty4 gene expression in prostate cancer. Using the MethPrimer software package for CpG islands identification (http://www.urogene.org/methprimer/) we have identified 3 CpG islands spanning from 1,100 nt upstream of the putative transcription start through the first exon into the intronic sequence (Fig. 3A). This led us to investigate the possibility of epigenetic inactivation at this locus. We performed methylation analysis on 7 pairs of matched normal and tumor prostate tissue samples and an additional 15 prostate cancer tissue samples. Initial analysis of CpG islands in the 5′-flanking region of the Sprouty4 of genomic DNA samples from the five pairs of matched normal and tumor prostate tissues showed methylation in the CpG island approximately 1 kb upstream of the putative transcription start site, hereafter referred to as the 5′-flanking CpG island (labeled A-I in Fig. 3A). No methylation was observed in the CpG island in the proximal promoter region,

therefore all subsequent methylation analysis was carried out on the 5′-flanking CpG island. Examples of the methylation analysis of individual normal and cancer DNA samples are illustrated in Figure 3B. The mean frequency of methylation at individual CpG dinucleotides in the DNA samples is summarized in Figure 3C. Genomic DNA samples from tumor tissues had a higher frequency of methylation, with the CpG dinucleotides labeled E–I having a greater than fivefold higher frequency of methylation. Because CpG dinucleotides E–I exhibited about the same frequency of methylation, we also analyzed the average frequency of methylation in these five CpG dinucleotides for each individual patient (Fig. 3D). The results show that the number of methylations of CpG dinucleotides E–I is significantly higher in tumor samples ($P \leq 0.001$, Mann–Whitney rank sum test). To determine if methylation was correlated with decreased Sprouty4 expression, we determined the level of Sprouty4 transcripts in the same matched normal and cancer tissue samples used in the methylation analysis by quantitative RT-PCR (Fig. 3E). To examine the relationship between Sprouty4 promoter methylation and Sprouty4 expression, we compared the proportion of cases with Sprouty4 promoter methylation that have Sprouty4 expression that is lower than matched normal tissue (11 of 14 cases) to the proportion of cases without promoter methylation that have low Sprouty4 expression (2 of 8 cases). This difference is statistically significant by Fisher exact test ($P = 0.026$). However, it should be noted that in two cases with low Sprouty4 expression (cases 9 and 29) there was no detectable methylation at these sites.

Treatment of LNCaP with 5-Aza-2′Deoxycytidine can Restore Sprouty4 Expression

We initially measured Sprouty4 expression in the three commonly used prostate cancer cell lines LNCaP, DU145, and PC3. LNCaP cells had quite low expression (2.9×10^{-4} transcripts/β-actin transcript) with DU145 having slightly higher expression (4.04×10^{-4} transcripts/β-actin transcript) and PC3 having high expression (2.0×10^{-2} transcripts/β-actin transcript). These relative RNA levels correlate with protein expression as determined by Western blotting in which LNCaP had essentially undetectable expression of Sprouty4 (see Fig. 5, below). Analysis of LNCaP cells revealed methylation of the Sprouty4 promoter, which was not seen in DU145 or PC3 cells (data not shown). To test the hypothesis that pharmacological modulation of methylation can reactivate gene expression [36], we treated LNCaP cells in various doses of the DNA methyltransferase inhibitor 5-aza-2′deoxycytidine (Aza dCR). Figure 4 indicates that treatment of LNCaP cells with Aza dCR (2.5 μM) led to over twofold increase in Sprouty4 mRNA expression. Overall, the in vivo and in vitro data demonstrate that methylation in the Sprouty4 gene is responsible for its downregulation in prostate cancer.

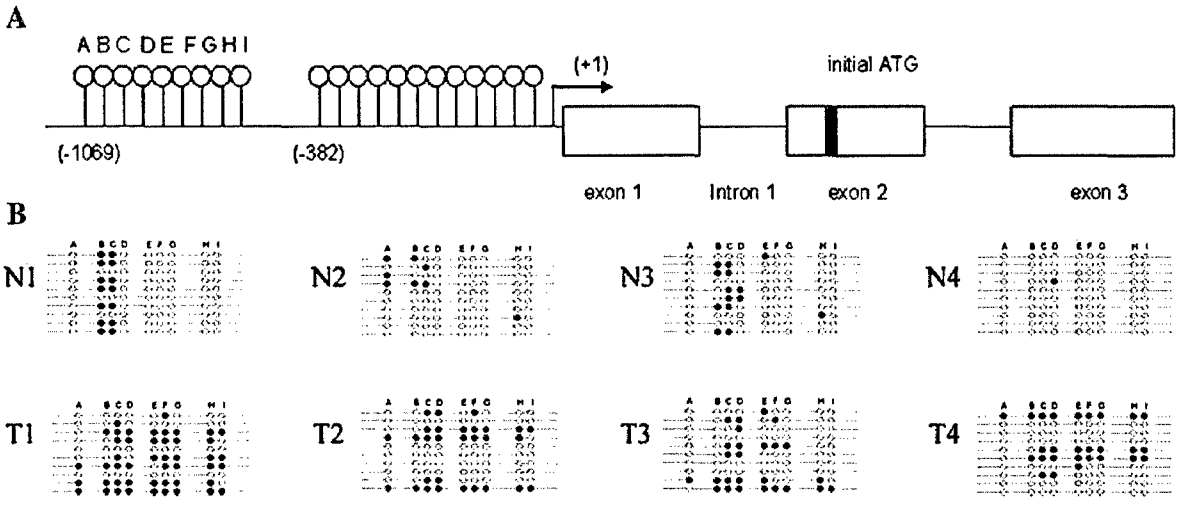

Fig. 3. Methylation analysis of the Sprouty4 gene. **A**: A schematic representation of the Sprouty4 gene. Distal CpG island is shown as 9 CpG dinucleotides (sites A–I). Transcriptional start (+1) site is shown as bar with an arrow-head. Exons are shown as open-boxes and translational start site shown as thick black bar. **B**: Analysis of CpG methylation in bisulfite-converted genomic DNA derived from four normal prostate tissue samples (N1–N4) and four cancer tissue samples (T1–T4). For each sample, 10 PCR clones were analyzed by sequencing. CpG methylated (black circle), CpG unmethylated (white circle). **C**: Frequency of methylation for each individual CpG dinucleotide from seven normal tissues (□) and 22 cancer tissues (■). **D**: Mean of methylation frequency of five CpG dinucleotides (sites E–I) in each individual patient. **E**: Ratio of Sprouty4 mRNA expression in cancer versus normal prostate tissue from each patient. Horizontal bar indicates equal expression in normal and cancer tissue samples.

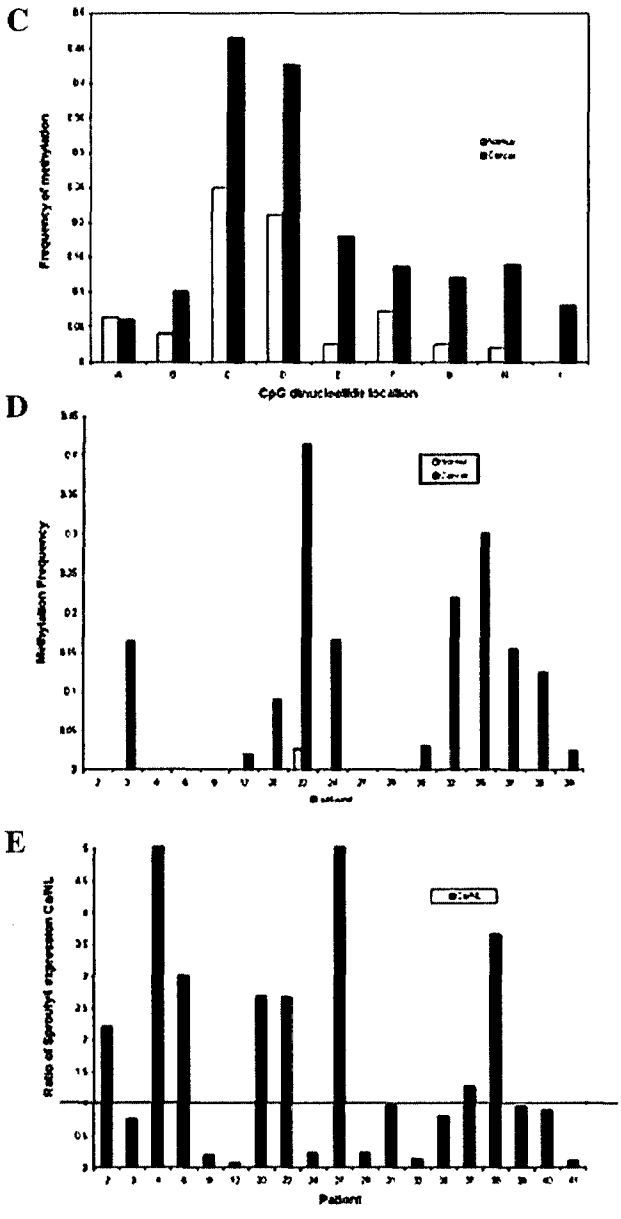

Fig. 3. (*Continued*)

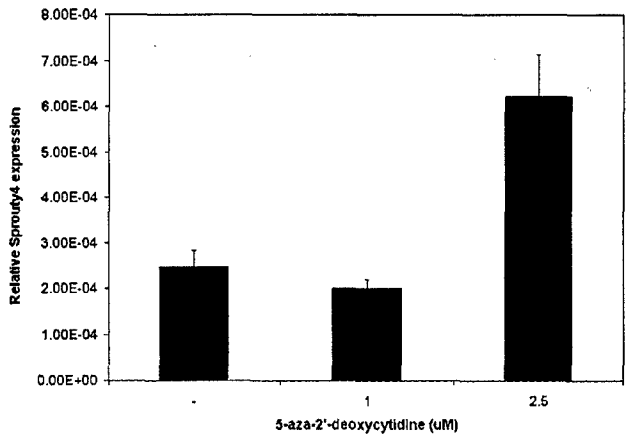

Fig. 4. Demethylation and Sprouty4 expression. Prostate cancer cell line LNCaP was treated with 5′-aza-2′-deoxycytidine (5′-aza-dC) at the indicated concentrations for 96 hr. Sprouty4 mRNA expression was determined by quantitative RT-PCR and expressed relative to β-actin to correct for variation in the amounts of reverse-transcribed RNA. The data is a representative of duplicate experiments.

The Biological Effect of Sprouty4 Over-Expression in Human Prostate Cancer Cells

To ascertain the biological effect of Sprouty4 expression in human prostate cancer cells, we transfected pcDNA-Sprouty4 (encoding the full length of Sprouty4 sequence) into human prostate cancer cell lines LNCaP, DU145, and PC3 and monitored cell proliferation. Transient expression of Sprouty4 in the prostate cancer cells did not seem to affect cell proliferation when compared the vector only control (Fig. 5A). To confirm the presence of Sprouty4 protein, these same cells were analyzed by Western blotting (Fig. 5B). The results shows an increase in Sprouty4 protein in both LNCaP and DU145 cells transfected with the Sprouty4 plasmid, however, we did not detect any significant increase of Sprouty4 protein in the PC3 which already has high basal Sprouty4 protein compared to the other two cell lines. We further verified the biological activity of Sprouty4 by transiently transfecting Sprouty4 plasmid into HUVEC. Transient transfection of HUVEC with Sprouty4 plasmid inhibited cell proliferation by 40% over 3 days when compared to cells transfected with the vector only control (data not shown). The result suggests that Sprouty4 protein mediated growth inhibition maybe cell-type dependent.

Increased cell migration and invasion is one of the characteristics associated with highly malignant phenotype of prostate cancer. To determine whether Sprouty4 protein inhibits prostate cancer cell migration, we over-expressed Sprouty4 in the DU145 prostate cell line, which shows modest basal Sprouty4 expression level (see Fig. 5B). The Du145 cells were transfected with Sprouty4 plasmid or vector only plasmid and several G418-resistant clones were selected and pooled together. We validated G418-resistant clones over-expressing Sprouty4 by Western blot analysis (Fig. 5C). In agreement with our transient transfection analysis, the stable over-expression of Sprouty4 in Du145 cells did not have any significant effect on cell proliferation (data not shown). To evaluate the effect of over-expressing Sprouty4 on Du145 cell migration, we utilized the scratch wound assay by assessing the rate of wound closure after scraping cells from an area of monolayer cultures.

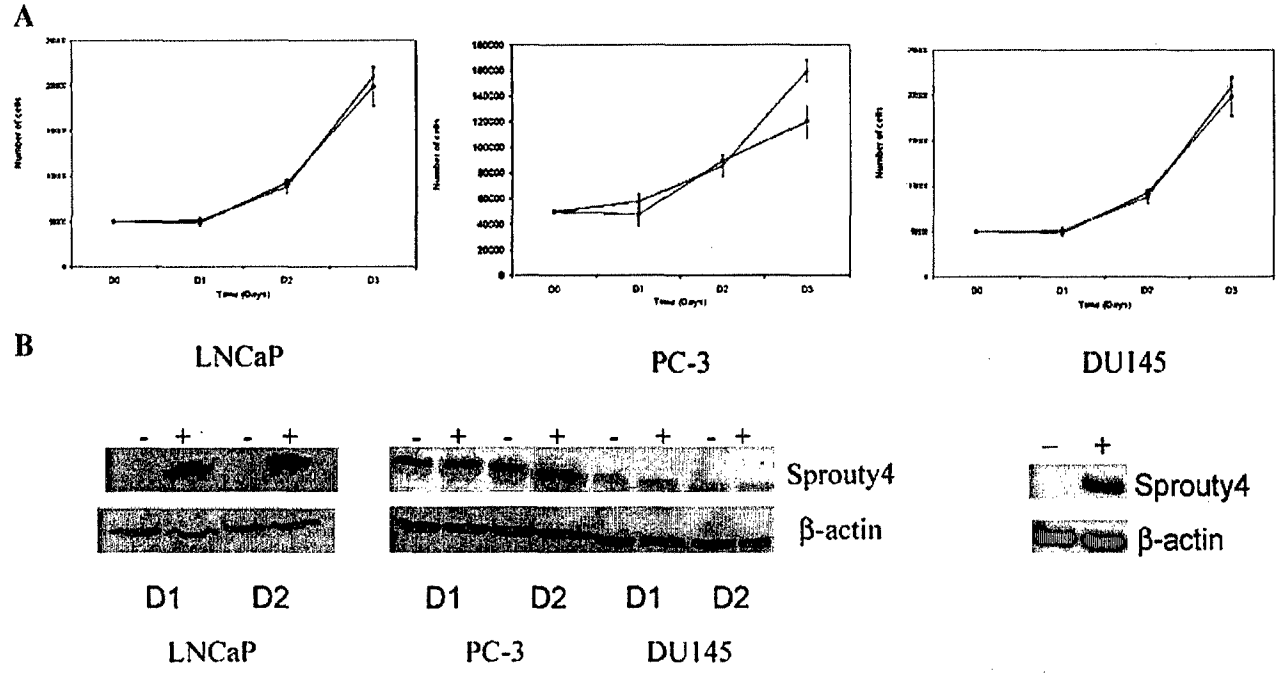

Fig. 5. Over-expression of Sprouty4 in prostate cancer cells. **A**: LNCaP, DU145, and PC-3 prostate cancer cell lines were each transfected with a Sprouty4 cDNA cloned in the mammalian expression vector pcDNA3.1 (■) or the pcDNA3.1 vector only (Δ). At the indicated times after transfection, cells were trypsinized and counted using a Coulter counter. All determinations were performed in triplicate and the standard deviation is shown. **B**: Protein extracts were collected from LNCaP, DU145, and PC-3 cells 1 and 2 days after transfection with pcDNA3.1 (–) or Sprouty4 cDNA in pcDNA3.1 (+) and analyzed by Western blotting with either anti-Sprouty4 antibody or control anti-β-actin antibody. **C**: DU145 prostate cancer cell line was stably transfected with pcDNA3.1 vector only (–) or pcDNA-Sprouty4 (+). After 2 weeks of selection in Geneticin, stable clones were selected, pooled together, and analyzed by Western blotting with either anti-Sprouty4 antibody or control anti-β-actin antibody.

Confluent DU145 cells were scraped and cells were allowed to migrate for 48 hr. As shown in Figure 6, control cells which were G418-resistant but not over-expressing Sprouty4 (vector control transfection) demonstrated higher rates of migration or wound closure when compared to cells over-expressing Sprouty4, which showed an obvious slower closer rate at the 24 and 48 hr time points. This experiment was replicated a total of four times with identical results.

DISCUSSION

In the present study, we were interested in understanding the role of Sprouty4 in prostate cancer and to elucidate the molecular mechanism regulating its expression. We have found by in situ hybridization and quantitative RT-PCR analysis that Sprouty4 is downregulated in the majority of human prostate cancers when compared to normal prostate tissue. Previously, we have shown that Sprouty1 is down-regulated in prostate cancer tissues [28] and that Sprouty1 can markedly decrease prostate cancer cell proliferation. Our data indicates distinct differences in the functional roles for Sprouty1 and Sprouty4 in prostate cancer cell lines. Transient expression of Sprouty1 significantly inhibited prostate cancer cell proliferation while stable over-expression was markedly deleterious to prostate cancer cells [28]. In contrast, transient expression of Sprouty4 did not have any significant effect on prostate cancer cell proliferation while stable over-expression of Sprouty4 inhibits prostate cancer cell migration. It has been shown that in some tissues the expression patterns of the Sprouty family members do not overlap [11]. These data indicate that the different isoforms of Sprouty are not uniformly regulated and suggests that the different family members may not be functionally equivalent. Thus the individual Sprouty genes may be regulated by specific combinations of factors to allow optimal control of signaling. It is likely that Sprouty4 mediates inhibition of cell migration in prostate cancer, at least in part, by repressing the kinase activity of TESK1. Sprouty4 has been shown to regulate the actin cytoskeletal reorganization by modulating the level of cofilin activity through TESK1 inactivation [22]. We have evaluated the expression of TESK1 by quantititative RT-PCR and TESK1 is expressed at similar levels in normal and neoplastic prostate tissues, as well as in

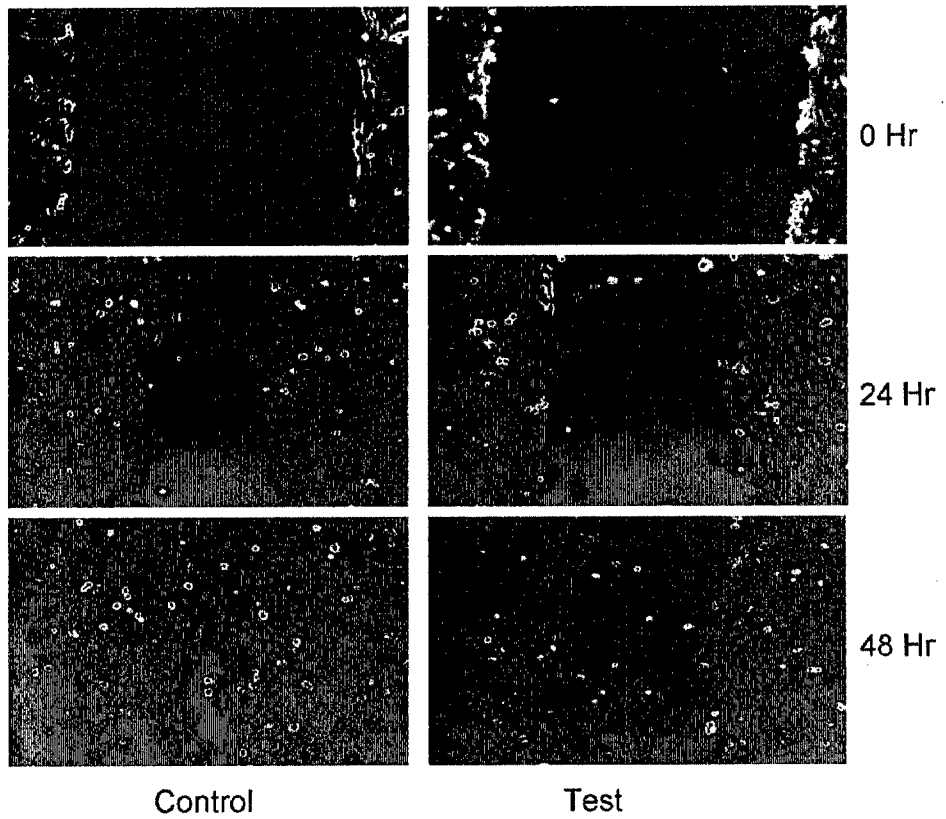

Fig. 6. Wounding assay of scatter/migration. DUI45 cells stably transfected with Sprouty4 plasmid (test) or vector only plasmid (control), were used in a scratch wound assay as described in Materials and Methods. The cells were permitted to migrate into the area of clearing for a total of 48 hr, and photomicrographs taken at 0, 24, and 48 hr. Results shown are typical of four separate experiments.

primary prostatic epithelial cells, immortalized normal prostatic epithelium (PNT1a), and all prostate cancer cell lines tested, namely PC3, DU145, LNCaP, and LAPC4 (data not shown). Thus TESK1 is present in prostate cancer cells and can be inactivated by Sprouty4 in the absence of Sprouty4 methylation. It should be noted that while the majority of prostate cancers have decreased Sprouty4 expression, some have robust expression, based on both in situ hybridization and quantitative RT-PCR. Given that FGFs can induce Sprouty gene expression in normal tissues and that multiple FGFs are expressed at increased levels in prostate cancer tissues [1] this would be the expected pattern in the absence of specific alterations in cancer cells affecting Sprouty4 expression. Further investigations are needed to determine whether Sprouty4 expression inhibits disease progression in this subset of prostate cancers.

Multiple genetic alterations can drive tumorigenesis and progression. The metastatic and drug/hormone-resistant phenotype of certain cancers such as prostate cancer may result from epigenetic events such as aberrant gene methylation [37–39]. Generally, aberrant gene methylation occurs at proximal promoter CpG islands [40]. However, the methylation of CpG islands several kilobases away from promoter region, typically in gene enhancer region, can also lead to aberrant gene expression as seen in genetic imprinting and also in cancer (for review see Bird [40]). In our studies we did not observe CpG methylation in the Sprouty4 promoter CpG island in five pairs of matched normal and tumor prostate tissue samples or in the LNCaP prostate cancer cell line. On the other hand, we observed that Sprouty4 was extensively methylated at the 5′-flanking CpG island (approximately 1 kb upstream of the putative transcription start site) in a subset of tumor tissues compared with matched normal prostate. Studies by Ding et al. [35] showed significant enhancement of Sprouty4 promoter activity in transient transfection assays when the region from 979 to 1,182-bp upstream from the transcription initiation site was included in the promoter constructs, suggesting the presence of an enhancer activity upstream of the core promoter region in the region containing the methylated CpG island that we have idemtified. The increase in methylation significantly correlated with the decreased Sprouty4 expression, as analyzed by quantitative real-time PCR, demonstrating that, in majority of cases, the down-

regulation of Sprouty4 in a prostate cancer cases is due to promoter methylation. However, two cases without detectable promoter methylation had low Sprouty4 expression. It is possible that failure to detect methylation reflects the well-known problem that the bisulfite modification technique followed by PCR, cloning, and sequencing, while considered the "gold standard" for quantitative analysis of methylation, is not 100% efficient in detecting methylation due to a number of potential problems, which may be gene specific, that limit its efficiency (for discussion see Dahl and Guldberg [41]). Other mechanisms of gene inactivation, such as alterations in trans-acting factors and heterozygous or homozygous deletion could also affect Sprouty4 expression and remain to be explored. Of note are the recent studies by McKie et al. [42] that have identified methylation of the Sprouty2 promoter in high-grade prostate cancer, which is correlated with decreased Sprouty2 expression. Thus more than one Sprouty gene is inactivated by methylation in human prostate cancer. Whether the Sprouty1 promoter is also methylated in prostate cancer is being actively investigated by our laboratory. However, the diverse biological roles of different Sprouty family members in prostate cancer in suggests that Sprouty signaling may provide an attractive new target approach for therapeutic intervention that may modulate a large number of potential growth promoting stimuli, including multiple growth factors and their receptors.

CONCLUSIONS

Sprouty4 expression is downregulated in human prostate cancer by DNA methylation and this decreased expression may contribute to increased cell migration.

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